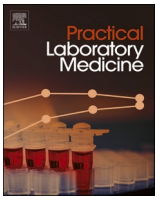
Practical Laboratory Medicine 42 (2024) e00433

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm



Registered Report Stage II

Validation of a LC-MS/MS assay for citric acid, cysteine and oxalic acid determination and its application to explore pre-analytical sample storage

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ARTICLE INFO

Keywords:
LC-MS/MS
Citrate
Oxalate
Cystine
Urinary store disease
Stability

ABSTRACT

Objectives: Citrate, oxalate and cystine in 24-h urine are considered to be associated with the incidence and recurrence risk of urinary stone disease (USD). An evaluation of the LC-MS/MS kit for simultaneous quantification of the three analytes was undertaken.

Design: & Methods: The analytical performance of the kit was investigated based on FDA, EMA and CLSI guidelines. To promote the standardization of sample storage, this kit has been applied to perform systematic pre-analytical stability study of these analytes in urine.

Results: This method was validated with good linearity with accuracy of 93.1%–104 %. Intra-day and inter-day imprecision were ≤5.55 % and 5.34 %, respectively. Recoveries of citrate, oxalate and cystine added to clinical samples were in the range of 92.0–103 %, 94.8–100 % and 99.0–107 % with CV ≤ 5.52 %. It was recommended that urine preserved with hydrochloric acid could be preferable in consideration of both reliable test results and neglected sample heterogeneity.

Conclusions: This kit is suitable for measurement of citrate, oxalate and cystine for understanding the etiology of urinary stones, and the proper storage of urine samples is crucial for the correctness of the test results.

1. Introduction

The history of urinary stones almost begins and goes parallel with the history of civilization, and the roots of modern science and the history of urinary stone disease trace back to the Ancient Egyptians and Mesopotamia [1]. Urinary stone disease (USD), also known

Abbreviations: USD, urinary stone disease; FT-IR, Fourier transform infrared; LC-MS/MS, liquid chromatography-tandem mass spectrometry; HPLC, high performance liquid chromatography; HCl, hydrochloric acid; QCs, quality controls; CVs, coefficient of variations; LLOQ, lower limit of quantification; LOD, limit of detection; MRM, multiple reaction monitoring; DP, delustering potential; CE, collision energy; CAP, College of American Pathologists.

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https://doi.org/10.1016/j.plabm.2024.e00433
Received 29 August 2024; Received in revised form 20 September 2024; Accepted 27 September 2024
Available online 30 September 2024

as urolithiasis, is a common urinary system disease, referring to the formation of stones in the urinary system (including kidneys, ureters, bladder and urethra). The incidence rate of USD varies in different regions due to different lifestyles and dietary habits [2]. In general, the prevalence in Western countries (5–9% in Europe, and 13 % in North America) is higher than that in Eastern countries (1–5% in Asia). In China, the overall incidence of USD is about 4 % of the population and increases steadily during the recent 20 years, due to high calcium intake and lack of exercise [3–5]. More importantly, USD is characterized by high recurrence rate. Early studies reported that within 5–10 years after the initial stone event, the recurrence rate of USD was up to 30%–50 %, which would lead to repeat or unplanned care [6,7]. As a result, effective prevention strategies and precise treatments are needed to reduce the burden caused by high prevalence of USD.

With the gradual deepening of human understanding of USD, it is known that defining stone composition is important for making a treatment plan, understanding etiology and preventing recurrence [8], which is helpful for investigating metabolic risk factors of USD. Although Fourier transform infrared (FT-IR) analysis of stone composition has been widely used in clinical practice, FT-IR is difficult ro detect minerals in low proportion, poor in information about the composition ratio of urinary stones, and requires experienced interpretation of spectra results [9,10]. Thus, direct metabolic assessment of urine is an important direction for understanding the etiology of urinary stones.

Considering the composition of urine stones, citrate, oxalate and cystine are of great significance in the diagnosis of USD. Citrate is usually present in urine and can inhibit the formation and growth of crystals. If the citrate content in urine is too low, it may increase the risk of stone formation. Low citrate levels are common in imbalances caused by certain metabolic diseases or dietary factors, which may indicate a potential tendency for stone formation [11]. It is well-known that calcium oxalate stones are one of the common types of stones accounting for about 80 %. Excessive oxalate content in urine may lead to the formation and precipitation of calcium oxalate crystals, thereby promoting the formation of stones. Detecting the oxalate level is helpful in determining whether stone formation is related to abnormal oxalate metabolism [12,13]. Cystine stones are relatively rare, but if an abnormally elevated cystine level is detected, it is specific for the diagnosis of cystine stones. As well-known, cystinuria is a genetic disease that leads to increased cystine excretion and is prone to the formation of cystine stones [14]. Thus, joint detection of these three analytes in urine, combined with the patient's clinical symptoms, imaging examinations, etc., can provide valuable information for the diagnosis of USD, analysis of the etiology and formulation of treatment plans. For example, if the oxalate level is significantly increased and the patient has stone symptoms and imaging evidence, calcium oxalate stones may be diagnosed, and further search for the causes of abnormal oxalate metabolism, such as dietary factors, intestinal diseases or certain genetic factors, should be performed. In conclusion, the detection of citrate, oxalate and cystine is an important link in the diagnosis and research of USD.

Traditionally, these analytes are always determined by enzymatic or spectrophotometry methods which have limited sensitivity and is susceptible to interference [15–17]. With the development of analytical techniques, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has attracted much attention for analysis of citrate, oxalate and cystine due to its high specificity, high selectivity, high sensitivity and high throughput [18–22]. Although LC-MS/MS technology can be used to provide tightly controlled analytical data, the lack of standardized methods and reagents allows large inter-laboratory differences to exist [23]. In addition, the stability of citrate, oxalate and cystine in urine is of great importance for test results, and a few studies have conducted systematic research on stability, especially on the types of preservatives.

Here, we report the result of evaluating a LC-MS/MS kit based on chemical derivatization for the quantitation of citrate, oxalate and cystine. The adoption of the kit may facilitate harmonization of these analytes measurement among laboratories. It is well-known that urine storage is more complex due to the alternative presevatives. We aimed to perform systematic pre-analytical stability studies for standardized sample preservation by this method.

2. Experimental

2.1. Reagents and chemicals

Kits were supplied by Beijing Health biotech Co. Ltd. (Beijing, China). The kit reagents consists of six vials of calibrators (citrate concentrations of 40.0, 80.0, 60.0, 300, 600, 1000 μg/mL; cystine concentrations of 4.0, 8.0, 16.0, 30.0, 60.0, 100 μg/mL; oxalate concentrations of 10.0, 20.0, 40.0, 75.0, 150, 250 μg/mL) and three vials of quality controls (QCs) (citrate concentrations of 120, 500, 700 μg/mL; cystine concentrations of 12.0, 50.0, 70.0 μg/mL; oxalate concentrations of 30.0, 125.0, 175 μg/mL) that prepared with synthetic urine. The kit are also equipped with sample processing reagents (derivative reagent A and B, acidifier, mobile phase additive A and B, and release agent C). The internal standard solution (citrate, cystine and oxalate concentration of approximately 50.0, 50.0 and 100 μg/mL respectively). High performance liquid chromatography (HPLC)-grade methanol was purchased form Merck KGaA (Germany). Hydrochloric acid (HCl, 37 %) and toluene were bought from Xilong Scientific Co., Ltd. (Guangdong, China). Diazoalkylurea was gotten from Macklin Biochemical Technology Co. Ltd (Shanghai, China). Deionized water was prepared by water purification system (Elga, England).

2.2. LC-MS/MS conditions

All analyses were performed on a Exion LC coupled with a Qtrap 5500 mass spectrometer (Applied Biosystems, AB Sciex, Foster City, CA, USA). The chromatographic separation was performed on a Shim-pack Cyslox column (50 × 2.1 mm, 2.7 μm, Shimadzu, Kyoto, Japan). The mobile phases consisted of solvent A (water with 0.2 % mobile phase additive A) and solvent B (methanol with 0.1 % mobile phase additive B). The flow rate was 0.4 mL/min and the column temperature was 40 °C. The separation was performed

under the gradient elution as follows: 0–0.20 min, 10%B; 0.21–0.70 min, 10%-80%B; 0.71–2.30min: 80%B; 2.31–2.90 min: 95%B; 2.91–4.50 min: 10%B. The injection volume was 1.0 μL. Between injections, the autosampler syringe was washed with 50 % methanol-water.

For MS analysis, the instrument was operated in positive ion mode with an electrospray ionization source as the following settings: ion spray voltage, 5500 V; temperature, 450 °C; curtain gas, 40.0 psi; collision gas: 8 psi; ion source gas 1, 55 psi; and ion source gas 2, 55 psi. The acquisition was achieved in the multiple reaction monitoring (MRM) scanning mode and two specific MRM transitions for each analyte were chosen. The specific MRM transitions, and important parameters including delustering potential (DP), collision energy (CE) and dwell time were shown in Table S1.

2.3. Sample preparation

At first, 1 mL of samples/calibrators/quality controls were acidified by 50 μL of acidifier. The above solution was further mixed and then centrifuged at 12000 rpm for 2 min. Secondly, 20 μL of the supernatant liquid was mixed with 10 μL of internal standard solution, and 200 μL of derivative reagent A and 100 μL of derivative reagent B were added in sequence. The tubes were capped, and the mixture was vortex-mixed for 10 s, and then incubated in a water-bath for 60 min at 60 °C. After the derivative reaction, the liquid is allowed to cool and then 0.3 mL of water was added. The obtained solution was mixed for 1 min and then centrifuged at 12000 rpm for 2 min. Finally, 20 μL of the upper layer was taken and mixed with 200 μL of release agent C in a 96-well plate. The microplate was shaken for 5 min, and then centrifuged at 4000 rpm for 5 min. The final solution was analyzed directly by LC-MS/MS.

2.4. Method validation

This method has been well validated according to FDA, EMA and CLSI C62-A in consideration of multiple parameters (imprecision, linearity, analytical sensitivity, accuracy, matrix effect, carryover, analytical specificity and stability).

2.4.1. Imprecision

Intra-day and inter-day imprecision were evaluated by measuring three quality controls in five independent batches with five replicates in each batch. Total coefficient of variations (CVs) were calculated as the square root of the sum of squared intra-day and inter-day CVs. The imprecision expressed as the CVs should not exceed 15 %.

2.4.2. Linearity

Linearity was evaluated by analyzing six-point calibrators. $1/x^2$ weighted linear regression model was fitted to the individual data. The determined concentration within ±15 % of expected concentration and the correlation coefficient (r^2) being ≥0.995 were accepted.

In addition, a series of five equally spaced dilutions were made from two pools, the concentration of which near to the low and high limits of the kit procedure. Then three replicates of each were analyzed for citrate, cystine and oxalate. According to CLSI EP 06, linearity was assessed according to 1st, 2nd and 3rd order polynomial regression analysis according to equation $Y = b0+b1X + b2X^2+b3X^3$.

2.4.3. Analytical sensitivity

The lower limit of quantification (LLOQ) was defined as the concentration which a pool of synthetic urine prepared with low concentrations of citrate, cystine and oxalate produced a CV within 20 % and a bias no greater than ±15 % while maintaining a signal-to-noise ratio above 10:1.

2.4.4. Accuracy

Recoveries were determined by addition of standard solutions of cortisol into the patient samples (1:9, v/v) at three concentrations (116.2, 457.2, 656.5 μg/mL for citrate, 12.1, 46.6, 65.8 for cystine, 30.2, 126.0, 178.0 for oxalate). Five replicates of spiked and non-spiked sample were analyzed for calculating CVs. The recovery was calculated as [(final concentration-initial concentration)/added concentration]. Recoveries are within 85%–115 % is accepted.

2.4.5. Matrix effect

The standard solution of the three analytes were mixed with at least six native urine specimens at a ratio of 1:1 (v/v). The matrix effect was evaluated by comparing the concentration obtained from the mixture *vs* the average concentration obtained by the standard solution and the urine specimen. The percentage differences within ±20 % were accepted.

2.4.6. Carryover

Carryover was evaluated by alternating injection of high concentration samples and blank samples six times. A method is free from carryover when the peaks associated with the blank samples should below the limit of detection.

2.4.7. Analytical specificity

By adding 0.52 g/L ascorbic acid (vitamin C) to the urine samples at a ratio of 1:9 (v/v) to simulate human samples with excessive ascorbic acid, six replicates are prepared in parallel. The interference of ascorbic acid is evaluated by comparing with the concentration

of the urine sample with and without addition of ascorbic acid ascorbic acid. Percentage difference, calculated as [(analyte concentrations in the spiked sample - analyte concentrations in the non-spiked sample)/analyte concentrations in the non-spiked sample] × 100 %, was employed to evaluate interference. Percentage difference within ±15 % was acceptable, indicating ignorable interferences.

2.4.8. Sample stability

Because a 24-h urinalysis needs a timed urine collection, standardized conditions for collection, preservation and storage of urine is important. We aim to explore sample stability under different conditions in detail. Morning spot urine samples were collected from volunteers into urine beakers. We refranined from collection of 24-h urine sample to obtain timepoint zero samples with different additives from one person. As well, 24-h urine samples preserved with hydrochloric acid (30 mL 6 M hydrochloric acid per 24-h collection), diazolidinyl urea (5 mL of 250 g/L diazolidinyl urea per 24-h collection) and methylbenzene (30 mL methylbenzene per 24-h collection) were gotten from addition of a certain amount of above preservatives into a fresh urine sample to simulate the effect of 24 h urine storage in different preservatives. At last, 24-h urine sample without preservatives and 24-h urine sample with hydrochloric acid, diazolidinyl urea and methylbenzene as a preservative were used for investigating sample stability under different storage conditions. The above fresh urine was aliquoted and stored at room temperature (RT) or in a refrigerator (4 °C) or freezer (−80 °C). The first set was assayed immediately and served as the reference point. The other aliquots were measured at least three times under different storage condition at each time point (day1, day 2, day 3, day 5, day 7 and day 14, respectively).

Meanwhile, a compare study about sample stability related to samples from different subjects under different conditions was performed in consideration of sample heterogeneity (n = 3). This work was approved by the ethics committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

2.5. External quality assessment

The quality of urinary citrate, oxalate and cystine assay has been evaluated by an external quality assessment from the College of American Pathologists (CAP). For CAP, three samples in each batch were sent every 6 months for testing and comparison with other labs were performed. Samples were sent on dry ice and stored at −80 °C until the specified data. Laboratories were encouraged to measure samples and were instructed to process the external quality control samples along with their own patient samples.

2.6. Data analysis

Data acquisition and processing were performed using Analyst software 1.6.2 (AB Sciex, Framingham, MA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3. Results

Following derivatization process and LC-MS/MS analysis, each analyte co-eluted with its corresponding internal standards as displayed in Fig. 1.

3.1. Method validation

3.1.1. Imprecision

Data to access intra-day, inter-day and total imprecision was collected with two quality controls. As demonstrated in Table 1, the intra-day and inter-day CVs for QCs were ranged from 0.75 % to 5.55 % and 2.97 %–5.34 %, while the total CVs of QCs were in the range of 3.33%–6.99 %.

3.1.2. Linearity

Linearity across the analytical measurement range was assessed. The best fit was achieved with square linear regression and a

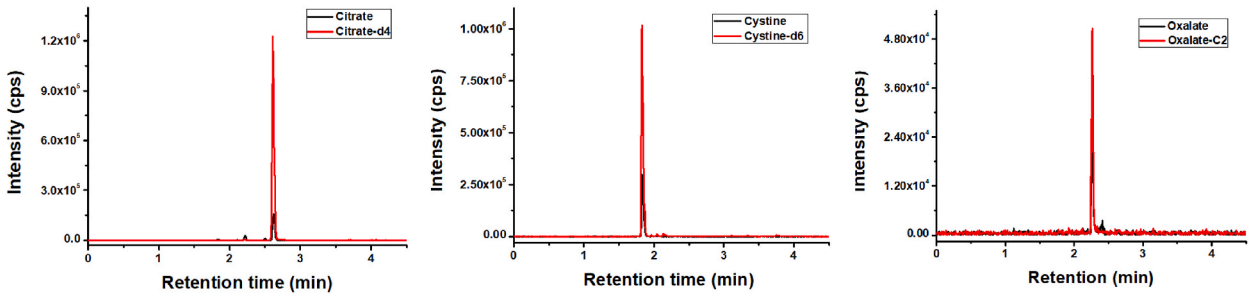

Fig. 1. The chromatograms of citrate (106 μg/mL), cystine (13.4 μg/mL) and oxalate (25.9 μg/mL) from a urine specimen.

weighting of $1/x^2$. The calibration curves were y = 0.0014x-0.0045 with linear correlation coefficient (r^2) of 0.9986 for citrate, y = 0.0231x-0.0022 with r^2 of 0.9986 for cystine, and y = 0.0233x+0.0255 with r^2 of 0.9988 for oxalate, respectively (Table 2). The deviation of the measured concentrations to the nominal concentrations at each calibration level were fulfilled the acceptance criteria of ±15 %. All these indicated the satisfying linearity with wide linear range.

In addition, A linear relationship best fit the citrate, cystine and oxalate concentration data was demonstrated using three replicates of five equally spaced dilutions a Low (231 μg/mL for citrate, 6.2 μg/mL for cystine and 15.3 μg/mL for oxalate) and a High (893 μg/mL for citrate, 68.7 μg/mL for cystine and 172 μg/mL for oxalate) urine pool, respectively. A good linear fit for each analyte was gotten as displayed in Fig. 2.

3.1.3. Analytical sensitivity

LLOQ, is determined as the lowest measurable concentration that gives a signal-to-noise above 10. The LLOQ was 40.0 μg/mL with a bias of 0.54 % and a CV of 2.37 % for citrate, 0.10 μg/mL with a bias of −12.60 % and a CV of 3.13 % for cystine, and 5.00 μg/mL with a bias of 13.45 % and a CV of 5.71 % for oxalate (Table 2).

3.1.4. Accuracy

Accuracy was investigated by evaluation of the performance of the kit procedure with regard to recovery of added analytes. A serial of clinical samples was supplemented with an additional standard solution of analytes at three levels. The recoveries ranged from 92.0 to 103 % for citrate, 94.8–100 % for cystine and 99.0–107 % for oxalate (Table 3) with CVs≤11.3 %, which achieved the acceptance criteria of 85–115 % recovery of the added analytes.

3.1.5. Matrix effect

Matrix effect is a common phenomenon in MS analysis, especially in usage of electrospray ionization source. Evaluation of matrix effect is significant in a LC-MS/MS method. Here, a mixture experiemnt was performed. In comparison with the average concentration calculated from the standard solution and the urine specimen, the pecentage differences of the mixture were in the range of 2.30–9.27 % for citrate, −2.99–2.85 % for cystine, and 2.23–8.97 % for oxalate (Table 4), indicating no obvious matrix effect.

3.1.6. Carryover and analytical specificity

No carryover was observed when a blank sample was injected after the high concentration sample. The peaks associated with the blank samples (LOD) were 6404 (114333) for citrate, 456 (9905) for cystine and 935 (13700) for oxalate all below LODs.

In consideration that ascorbic acid is the main precursor for oxalate production in human body, and calcium oxalate is the most common stone composition, the potential interference from ascorbic acid has bee studied. However, the percentage differences were −4.05 % for citrate, −3.84 % for cystine and −3.27 % for oxalate as shown in (Table S2), indicating no obvious interference was observed after addition of ascorbic acid.

3.1.7. Stability

Because urine samples determined by LC-MS/MS method always cannot be pretreated immediately in a clinical laboratory, thus it is necessary to evaluate sample stability. To this end, mimic 24-h urine with different preservatives were stored at room temperature, 4 °C and −80 °C and measured citrate, cystine and oxalate concentrations for up to fourteen days. The EMA and FDA guidelines indicate that analyte stabilities in the samples should be within ±15 % of the values at t = 0 with freshly preparation and time period [24,25]. The sample stability was investigated based on this criterion, Under storage at rome temperature without preservative, compared with initial concentrations, citrate decreased by more than 15 % on day 5, and loss of citrate was 57.48 % after one week and 94.98 % after 14 days, but could be stable at 4 °C and −80 °C with average percentage differences in the range of −7.64%-5.32 % (Fig. 3). For citrate, urine samples preserved with diazoalkylurea, HCl and toluene did not show a significant change when stored at room temperature, 4 °C and −80 °C for 14 days (Fig. 3). Cystine in urine without preservative is not stable at room temperature, and could be stored at 3 days, 7 days and 1 days with diazoalkylurea, HCl and toluene as a preservative, respectively. At 4 °C and −80 °C, whether preservatives are added or not, cystine in urine was relative stable after 14 days (Fig. 4). With regard to oxalate, the loss of oxalate in native urine appeared on day 3 at both room temperature and 4 °C. It was also not stable when using toluene as a preservative except at 4 °C (Fig. 5). Diazoalkylurea and HCl seem to be relatively suitable preservatives for oxalate in urine due to the

Table 1
Evaluation of the imprecision of the Kit procedure.

Analyte	QCs	Mean concentration (μg/mL)	Intra-day CV (%)	Inter-day CV (%)	Total CV (%)
Citrate	Low	128.23	1.90	4.41	4.80
	Medium	502.87	1.51	2.97	3.33
	High	704.93	4.63	3.88	6.04
Cystine	Low	13.07	0.75	5.34	5.39
	Medium	50.23	3.10	4.07	5.12
	High	69.68	3.86	3.80	5.42
Oxalate	Low	33.19	1.62	4.71	4.98
	Medium	126.10	4.43	3.42	5.60
	High	174.87	5.55	4.25	6.99

Table 2
Linearity and LLOQ.

Analyte	Linear range (μg/mL)	Linear equation	r^2	Accuracy	LLOQ (μg/mL)
Citrate	40–1000	y = 0.0014x-0.0045	0.9986	93.1%–103 %	40.0
Cystine	4–100	y = 0.0231x-0.0022	0.9998	98.2%–102 %	0.10
Oxalate	10–250	y = 0.0233x+0.0255	0.9988	96.0%–104 %	5.00

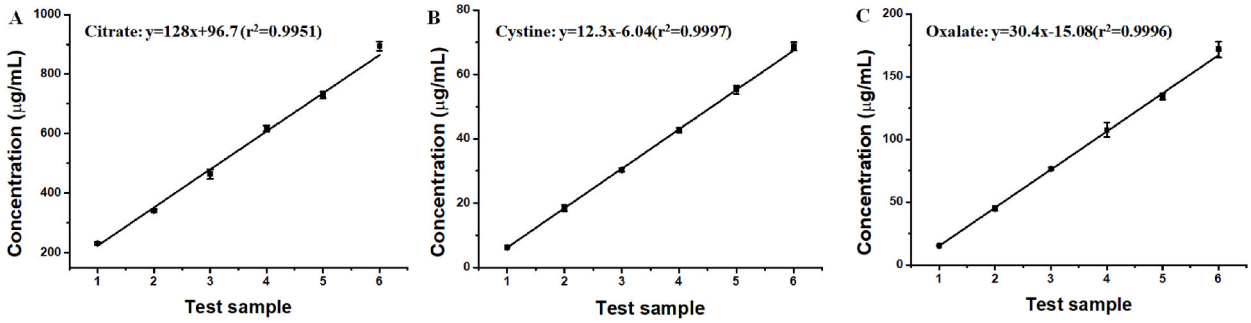

Fig. 2. Linearity study-Dilutions.

Table 3
Recovery.

Analyte	Spiked concentration (μg/mL)	Recovery (%)	CV (%)
Citrate	116.2	92.0	11.0
	457.2	103	2.25
	656.5	99.4	4.93
Cystine	12.1	94.8	11.3
	46.6	100	4.08
	65.8	99.1	5.33
Oxalate	30.2	107	5.18
	126.0	105	5.39
	178.0	99.0	5.52

Table 4
Matrix effect.

Urine lot	Percentage difference (%)		
	CA	Cys	OA
Lot 1	9.27 %	2.85 %	4.68 %
Lot 2	5.43 %	2.37 %	2.78 %
Lot 3	3.84 %	−0.62 %	3.35 %
Lot 4	2.30 %	−2.99 %	2.23 %
Lot 5	4.66 %	−0.01 %	8.97 %
Lot 6	2.99 %	−0.09 %	5.43 %

satisfying stability under different conditions. To sum up, HCl is a preferable preservative.

Considering the heterogeneity among the samples, the stability of urine samples from different subjects was further investigated (Fig. S1/S2/S3). For citrate, cystine and oxalate in urine without preservatives, they become unstable at room temperature within or after one day in some individuals, but they have almost the same stability trend at 4 °C and −80 °C, and remains stable for at least 2 days. For citrate, cystine and oxalate in urine with preservatives, HCl is the best preservative. When hydrochloricacid is used as a preservative, citrate, cystine and oxalate are relatively stable under various preservation conditions, and the stability change trend is similar in different subjects. However, diazoalkylurea is not favable for oxalate storage at room temperature more than 3 days, while citrate, cystine and oxalate are unstable after one day in some indiviuals using toluene as a preservative. In consideration of sampe heterogeneity, HCl is also recommended as an additive for urine storage, and the proper sample storage is significant to ensure the reliability of test results.

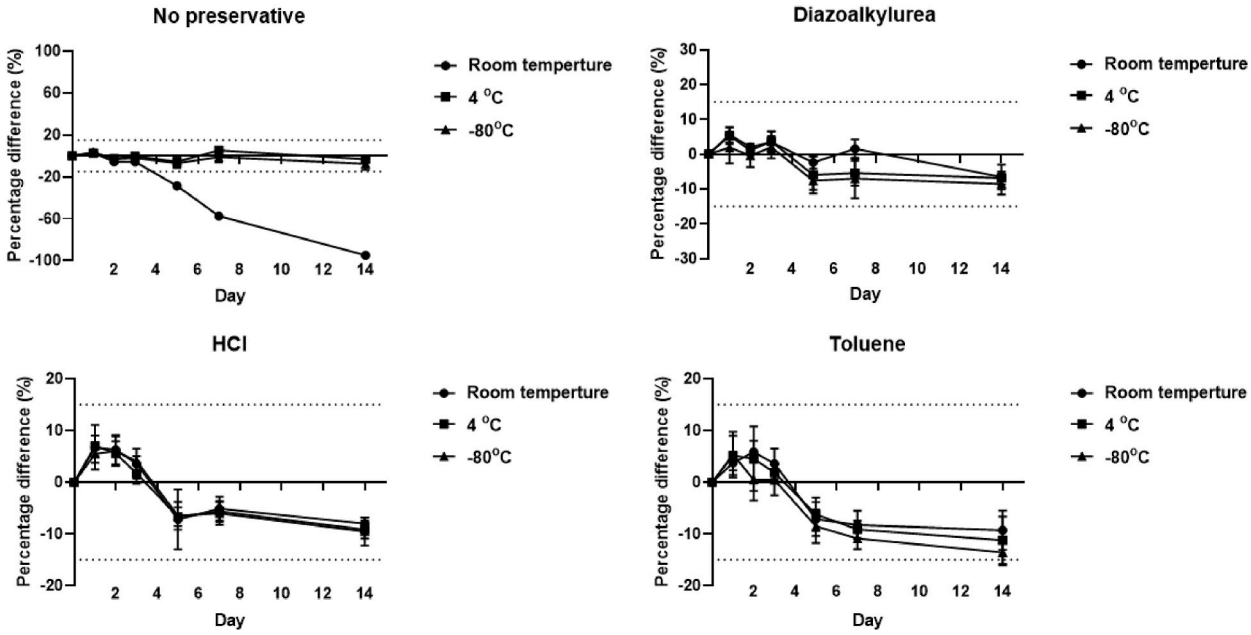

Fig. 3. Stability of citrate in urine samples over a 14 day period with and without preservatives under different storage conditons.

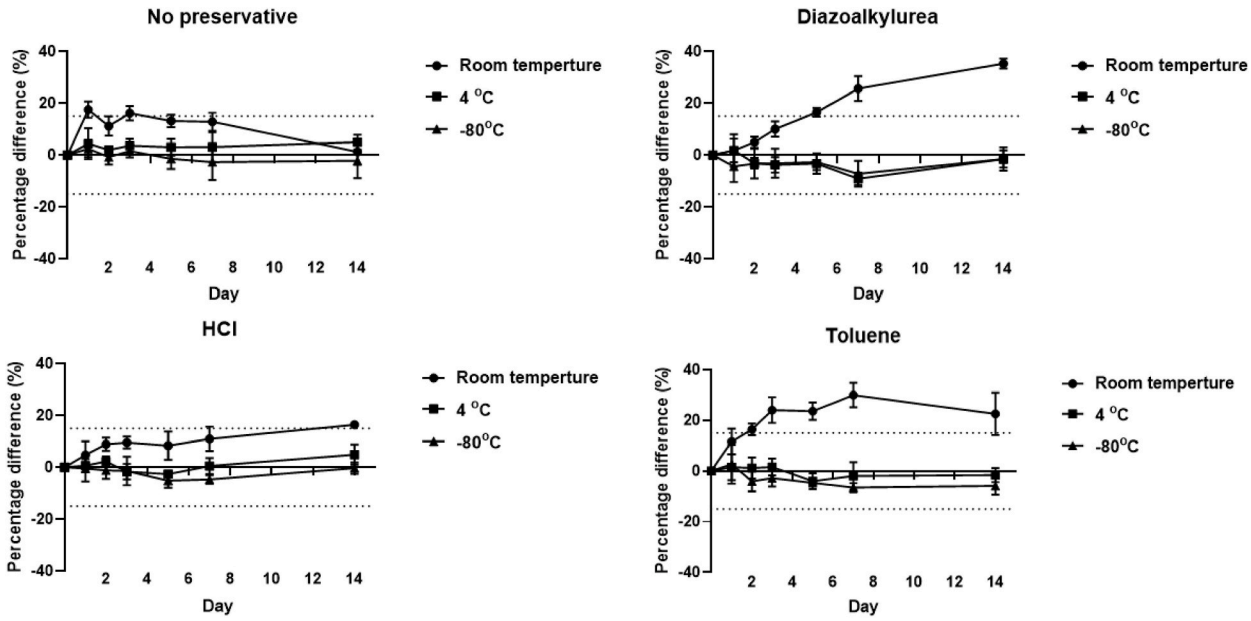

Fig. 4. Stability of cystine in urine samples over a 14 day period with and without preservatives under different storage conditons.

3.2. External quality assessment

In order to ensure the reliability of the test results, an external quality assessment from CAP was taken. In most laboratories, enzymatic or spectrophotometry methods have been used for determination of citrate and oxalate, but cystine has been measured by LC-MS/MS or HPLC (Table S3). The percentage differences of citrate and oxalate between our test results and target values form peer group mean were in the range of −13.41–22.12 %, and all results were within ±3SD based on the evaluation criteria from CAP (Table S3). Although cystine was not graded, but the percentage differences ranged from −9.31–18.18 %, indicating comparable test results of this LC-MS/MS method.

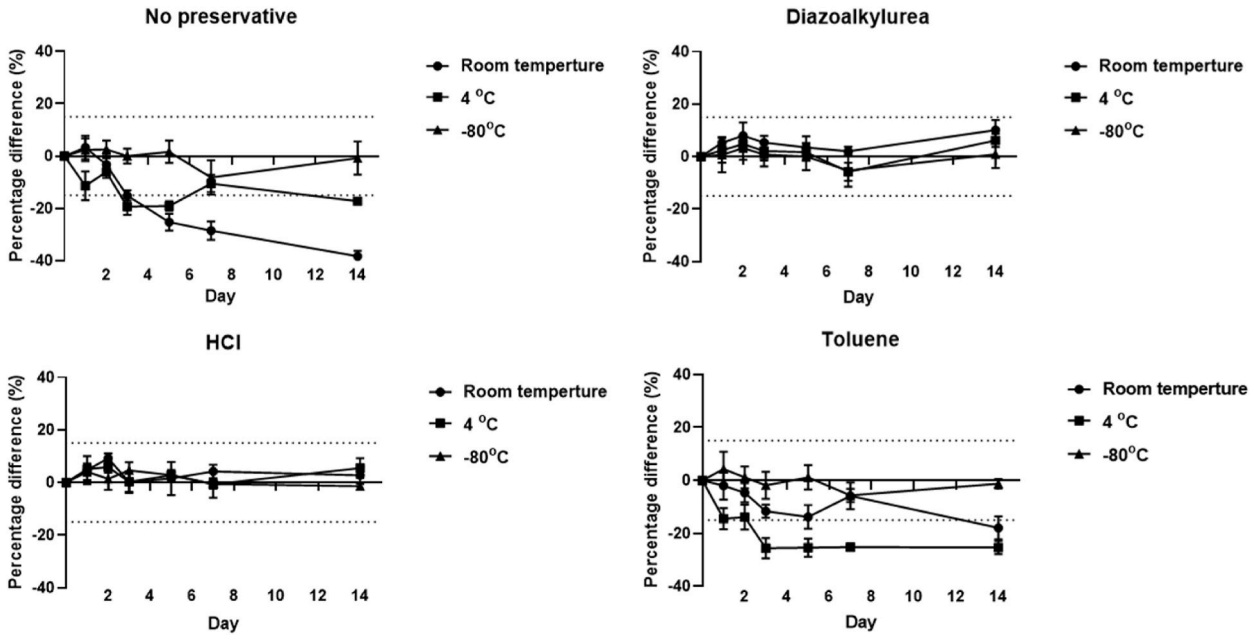

Fig. 5. Stability of oxalate in urine samples over a 14 day period with and without preservatives under different storage conditons.

4. Discussion

The prevalence of USD is about 4 % in China and increases steadily in recent years. Defining the stone composition is significant for the treatment and prevention of USD, Although FT-IR has been widely used in clinical practice for the analysis of stone composition, it is hard to determinethe proportion of the various compositions. Thus, direct metabolic assessment is an important direction for understanding the etiology of urinary stones.

Rencent trends suggest that LC-MS/MS is favored over other semi- and automated platforms and has been chosen as a promising method for analysis of small molecules [26]. Recent CAP data indicate that LC-MS/MS procedures are rarely applied to determine citrate, cystine and oxalate, which might be due to their low molecular weight, high polarity, and limited stability [27,28]. As a result, the well-developed LC-MS/MS method for citrate, cystine and oxalate determination is very important. The requirement to develop and validate an in-house LC-MS/MS method, to source the necessary reagents and to prepare calibrators with consistent quality has sometimes acted as obstacles to adopt this technique in routine laboratories [23]. As a result, by offering a carefully validated method comparable to conventional assays, this kit will enable routine laboratories to begin adopting mass spectrometry. In addition, problem-solving requires a high level of skills but the use of a standardized procedure could simplify technical support. The evaluated kit provides a well-controlled procedure, eliminates the barrier of a home-made method, and provides the recommended procedures. This kit procedure achieves excellent performance. Good linearity (r^2 above 0.998), nice intra-day and inter-day imprecision ($\leq$5.55 % and 5.34 %), satisfying recoveries (92.0–107 %) and negligible matrix effect, ensuring the comparable results with other laboratories in proficiency testing from CAP (Table S3).

Apart from the method itself, the correct preservation and transportation of samples are also important aspects to ensure the accuracy of test results. Therefore, the stability of mimicked urine in different preservatives was studied in detail. Citrate in urine without preservatives is only stable at room temperature for up to 3 days, and it exhibited great sample heterogeneity as described in Kavanagh's study [29]. However, urinary citrate was stable in other storage conditions, especially when preserved by HCl. Cystine and oxalate in urine without preservatives is not stable when store at room temperature, and the situation varies among different people, which might contribute to the in inconsistency with the literatures [28,30]. However, in comparison with diazoalkylurea and toluene, citrate, cystine and oxalate are relatively stable under various preservation conditions when using HCl as a preservative. As result, HCl is recommended as an additive for urine storage, and the suitable sample storage is of great significance for the reliability of test results.

Limitations of the study include the small sample size. In addtion, due to the difficulty in collection of 24-h urine specimen with different preservatives from one person for comparative study, mimic 24-h urine specimens with various preservatives were used.

5. Conclusions

In summary, a LC-MS/MS Kit was evaluated with satisfactory performance verification results. The urine sample contaning citrate, cystine and oxalate could not be stable at room temperature among individuals, but at least 14 days at 4 °C and −80 °C if HCl was chosen as the proper preservative. Investigating the stability of urine samples is important for safe storage of samples prior to instrumental analysis.

CRediT authorship contribution statement

Ying Shen: Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Xia Luo:** Investigation, Data curation. **Qing Guan:** Supervision. **Wenjie Lou:** Resources. **Liming Cheng:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2024.e00433.

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